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Cold-acclimation and root temperature protection from chilling injury in chilling-sensitive mungbean (*Vigna radiata* L.) seedlings

Mon-Yaw Chang, Swu-Ling Chen, Chung-Fen Lee, and Yih-Ming Chen*

Department of Botany, National Taiwan University, Taipei 106, Taiwan, Republic of China

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Abstract. Exposure of mungbean seedlings to 4°C for 2 days induced irreversible chilling injury. The major cation in the leakage from tissues of unacclimated seedlings was K^+, the loss of which was 7 to 10-fold greater than that of Ca^{++} or Mg^{++}. Acclimation of seedlings at 10°C protected them from the injuries caused by the 4°C treatment. Acclimation of seedlings at 10°C for 2 to 3 days, significantly decreased the conductivity and the concentration of soluble sugars, free amino acids, and cations (K^+, Mg^{++} and Ca^{++}) in the leakage. Compared to the 28°C-root/28°C-shoot control seedlings, those in the 28°C -root/4°C -shoot treatment did not suffer noticeable injury, but seedlings in the 4°C-root/4°C-shoot treatment did. The solute potential, water potential, and the concentration of free amino acids and cations (K^+, Mg^{++}, and Ca^{++}) in the cell sap of the 28°C-root/4°C-shoot seedlings were similar to those of the control seedlings.

Keywords: Cell sap; Chilling injury; Cold acclimation; Conductivity; Leakages; Mungbean (*Vigna Radiata* L.); Root temperature.

Introduction

Tropical and subtropical plants exhibit marked physiological and biochemical dysfunctions, commonly referred to as chilling injury, when they are exposed to temperatures below l0°C to l2°C (Graham and Patterson, 1982; Wang, 1982; Guy, 1990). These dysfunctions include alteration in membrane structure and lipid composition (Lyons and Raison, 1970), metabolic modifications (Sochanowicz and Kaniuga, 1979; Levitt, 1980; Trevanion et al., 1995), changes in protein content (Marmiroli et al., 1986; Bredenkamp and Baker, 1994) and enzyme activities (Byrd et al., 1995; Kumar and Triphathy, 1998), phosphorylation of thylakoid proteins (Bannett, 1991), cyclosis (Lewis, 1956), redistribution of intracellular calcium ions (Bush, 1995), cellular leakage of electrolytes and amino acids, and a diversion of electron flow to alternate pathways (Leopold and Musgrave, 1979). Dysfunctions associated with chilling stress in mungbean seedlings may be attributable to the alteration of gene expression (Guy et al., 1985; Mohapatra et al., 1989; Kurkela and Franck, 1990; Ouellet et al., 1993; Wolfraim and Dhindsa, 1993; Wolfraim et al., 1993; Hughes and Dunn, 1996; Kung et al., 1998). However, there is still a paucity of information on the effects of cold acclimation and root temperature on chilling injury. In this study, we provided protection from chilling injury in mungbean seedlings using cold acclimation and warm root treatments.

*Correspondence author. Tel: 02-23630231 ext. 2360; Fax: 02-23919096; E-mail: yihmingc@ccms.ntu.edu.tw

Materials and Methods

Plant Material

Mungbean (*Vigna radiata* L.) seeds were immersed in running water overnight and then planted in vermiculite in a growth chamber set at 28°C and 45 μmol m^{-2} sec^{-1} (14L/10D). The seedlings were grown for 5 days under these conditions and then separated into two groups. Seedlings in the chilling treatment group were transferred to 4°C and 45 μmol m^{-2} sec^{-1} for various lengths of time. The control group seedlings were maintained at 28°C and 45 μmol m^{-2} sec^{-1}. For the cold-acclimation process, 5-day-old seedlings were subjected to l0°C for various lengths of time and then transferred to 4°C. To study the effect of root temperature on chilling injury, seedlings were grown at either 28°C or 4°C in a water culture containing Hoagland solution (Epstein, 1972).

Procedures

Preparation of cell sap from leaf discs and measurement of conductivity. Cell sap was obtained from frozen and thawed leaf discs as described by Zudo et al. (1983). The conductivity of electrolyte leakage in the cell sap from leaf discs was used as a measure parameter of chilling injury (Sukumaran and Weiser, 1972).

Quantitative analysis of soluble sugars, amino acids, proteins and cations. The concentration of amino acids that leaked from leaf discs into the incubation medium was determined by the ninhydrin methods described by Moore and Stein (1954), using leucine as a standard. Soluble sugars in the incubation medium were measured by the

phenol-sulfuric acid reaction of Dubois et al. (1956), using glucose as a standard. The protein content was determined using the methods of Lowry et al. (1951).

Amino acid composition in the cell sap was measured with an amino acid analyzer (LKB, Model 4150) and cations were analyzed by atomic absorption/flame emission spectrophotometry (Shimadzu AA-Model 690).

Measurement of water potential and solute potential. The water potential of leaf discs (1 cm in diameter) punched from leaves was measured with a microvoltimeter (Wescor HR-33t). Solute potential in the cell sap was measured with a Wescor Osmometer (Model 5100 C).

Results

Effects of Cold Acclimation on Leakage

Three days of acclimation at 10°C maximized mungbean seedling tolerance to chilling at 4°C (figure not shown). Acclimation significantly decreased the leakage of solutes and cations from the leaves of seedlings chilled at 4°C. Acclimation at l0°C for 2 to 3 days significantly decreased the conductivity (Figure 1), the concentration of soluble sugars and free amino acids (Figure 2), and the concen-

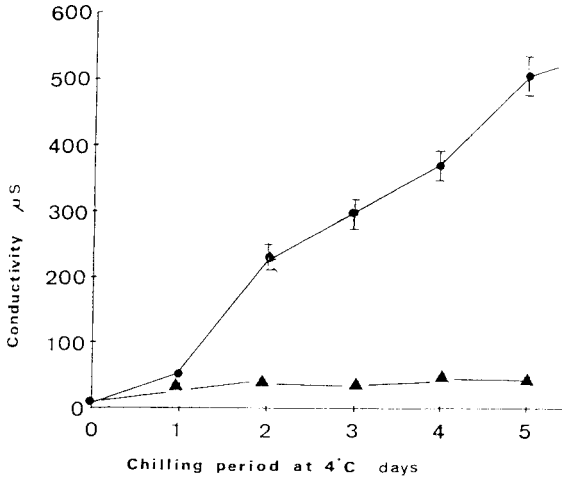

Figure 1. Solute leakage from mungbean leaves subjected to continuous chilling at 4°C versus 10°C -cold acclimation for 2 days followed by incubation at 4°C for various lengths of time. Five day-old mungbean seedlings were treated at 10°C for 2 days followed by incubation at 4°C for various length of time. Leaves (0.5 g) from the temperature treated-seedlings were incubated in 10 ml of deionized water for 2 h, and then the conductivity of the leakage in the medium was measured. Vertical lines represent standard errors (n=3). -●- 4°C; -▲- 10°C cold acclimation.

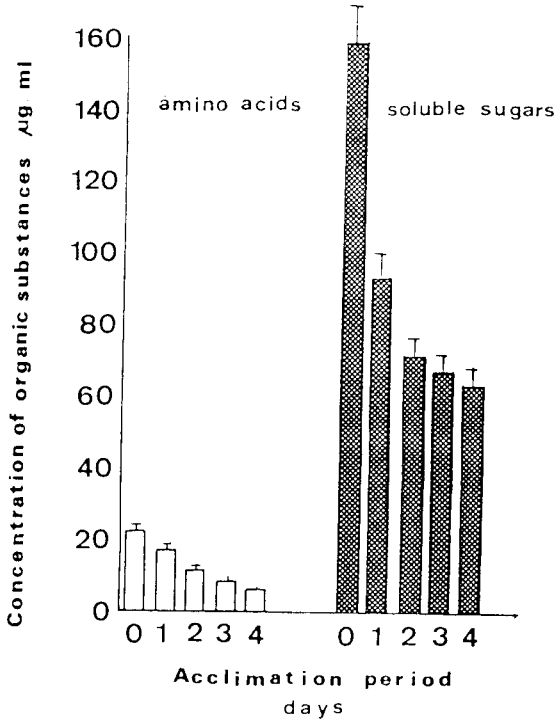

Figure 2. Effect of cold acclimation pretreatment on the amino acid and soluble sugar contents in the leakage from leaf discs. Five day-old mungbean seedlings were treated at 10°C for various lengths of time and then chilled at 4°C for 2 days. Leaves (0.5 g) from the temperature treated-seedlings were incubated in 10 ml of deionized water for 2 h. The amino acids and soluble sugars in the leakage of leaf discs were measured. In the control seedlings, the concentration of amino acids was 60 μg/g fresh weight and the concentration of soluble sugars was 480 μg/g fresh weight. Vertical bars represent standard errors (n=3).

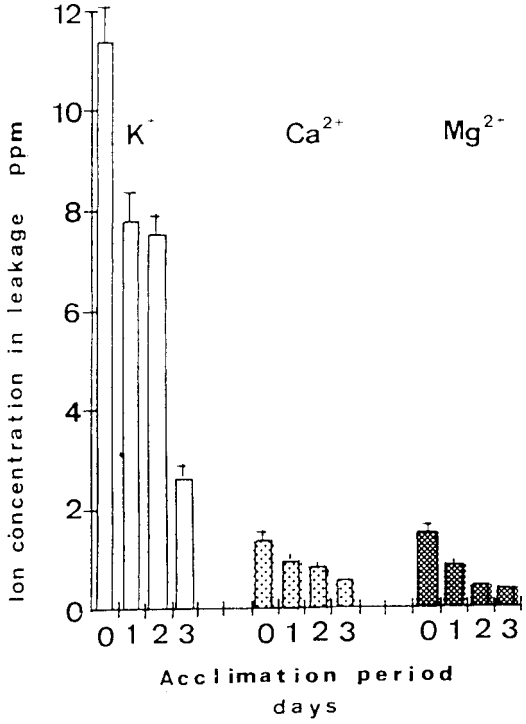

Figure 3. Effect of cold acclimation pretreatment on the K^+, Mg^{++} and Ca^{++} contents of leaf disc leakage. Five day-old mungbean seedlings were treated at 10°C for various lengths of time and then chilled at 4°C for 2 days. The concentrations of K^+, Mg^{++} and Ca^{++} in leakage of leaf discs were determined by atomic absorption/flam emission spectrophotometry. The concentrations of K^+, Mg^{++} and Ca^{++} in the leakage from the 28°C control plants were 2.12 ppm, 0.2 ppm and 0.3 ppm, respectively. Vertical lines reprent standard errors (n=3).

tration of cations (K^+, Mg^{++} and Ca^{++}) (Figure 3) in the leakage. The major cation in the leakage from tissues chilled at 4°C was K^+, the loss of which was 7 to 10-fold greater than that of Ca^{++} or Mg^{++} (Figure 3).

Quantitative Changes in the Solutes in Leaf Disc Cell Sap After Cold Acclimation

The concentrations of free amino acids and soluble sugars in the cell sap of 4°C-chilled seedlings decreased about 50% compared to those in the cell sap from control seedlings. However, the cold acclimation treatment caused an increase in the concentration of these two solutes (Figure 4). The cell sap of cold acclimated seedlings contained about 20% and 60% higher concentrations of soluble proteins than the cell sap of 4°C -chilled and control seedlings, respectively (Figure 5).

The cell sap of chilled, unacclimated mungbean seedlings contained l0 amino acids, lysine, histidine, aspartic acid, threonine, serine, proline, glycine, alanine, tyrosine, and glutamine, that increased slightly, and 6 amino acids, arginine, glutamic acid, valine, isoleucine, leucine and phenylalanine, the concentrations of which decreased slightly compared to their concentrations in the cell sap of control seedlings (Table l).

Table 1. Concentration of free amino acids in the cell sap of leaf discs from unacclimated and 10°C-cold acclimated mungbean seedlings. The experimental procedures are the same as in Figure 4. Concentration of major free amino acids in the cell sap of leaf discs were measured by amino acid analyzer. Data are expressed as means ± SE (n=3).

Amino acids	Acclimated	Unacclimated
	(nmole/g f.w.)	
Lys	278.8±14.9	237.8±8.7
His	899.8±46.1	710.5±36.3
Arg	270.5±10.3	324.8±13.2
Asp	1171.1±68.1	890.3±48.6
Thr	301.6±13.0	262.2±9.2
Ser	585.1±26.2	339.3±13.5
Glu	1730.5±97.0	2163.4±93.1
Pro	414.0±16.3	348.3±14.0
Gly	118.6±4.5	trace
Ala	2536.4±148.2	2172.1±105.4
Val	1305.5±64.2	1389.1±57.3
Ile	764.5±26.6	767.6±31.9
Leu	541.0±23.3	562.6±19.7
Tyr	127.0±15.4	95.7±3.2
Phe	113.2±4.3	124.7±3.4
Gln	590.2±26.8	368.3±11.7

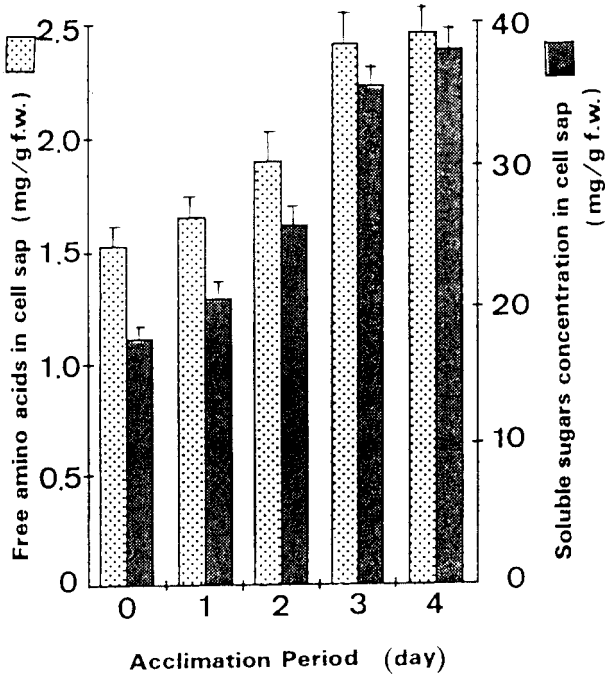

Figure 4. Free amino acid and soluble sugar concentrations in the cell sap of leaf discs after the 10°C cold acclimation treatment. Five day-old mungbean seedlings were treated at 10°C for various lengths of time and then chilled at 4°C for 2 days. The concentration of free amino acids and soluble sugars in the cell sap of leaf discs were measured. The concentrations of free amino acids and soluble sugars in the 28°C control seedlings were 3.38 mg and 15.2 mg per gram of fresh weight, respectively. Vertical lines represent standard errors (n=3).

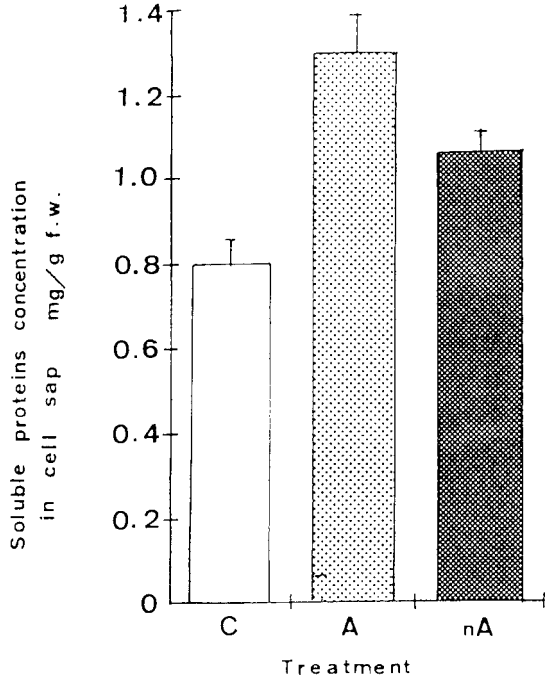

Figure 5. Concentration of soluble proteins in the cell sap of leaf discs from mungbean seedlings subjected to different temperature treatments. Five day-old mungbean seedling were treated at 10°C for 2 days. The soluble proteins in the cell sap of leaf discs from different temperature treatments were measured. Vertical lines represents standard errors (n=3). C: Control (28°C, 7d); A: Acclimated (28°C, 5d → 10°C, 2d → 4°C, 2d); nA: Unacclimated (28°C, 5d → 4°C, 2d).

Compared to the controls, the Ca^{++} concentration increased in the cell sap of unacclimated seedlings, but decreased in the cell sap of acclimated seedlings. Both treatments caused a decrease in K^{+}, but neither treatment affected the concentration of Mg^{++} (Figure 6).

The water content and leaf water potential of mungbean seedlings decreased following exposure to temperatures lower than 10°C (Figure 7). However, acclimation caused an increase in the concentration of soluble sugars, free amino acids, and soluble proteins in the cell sap (Figure 4 and 5), lowering the osmotic potential in the tissues (Figure 7). Consequently, the seedlings had more water content and were more resistant to chilling injury at 4°C.

Effect of Root Temperature on Shoot Injury

When the shoot portion of seedlings was exposed to 4°C and the roots were kept at 28°C for 2 days, there was noticeable chilling injury of the leaves (figure not shown). The conductivity of leaf cellular leakage obtained from the 28°C-root /4°C-shoot treated seedlings decreased about 76% compared to that of whole seedlings kept at 4°C. The solute potential and water potential in the 28°C-root /4°C-shoot seedlings were similar to those of the control seedlings (Table 2).

Effects of Root Temperature on the Concentration of Free Amino Acids, Soluble Sugars, and Cations in the Cell Sap of Leaf Discs

When the root temperature was kept at 28°C and the shoot was exposed to 4°C, the concentration of free amino acids and cations (K^{+}, Mg^{++} and Ca^{++}) in the cell sap remained at the same level as in the control seedlings (Table 3).

Discussion

The leaf disc leakage of solute into the incubation medium are thought to be controlled by the plasma membrane. Increased leakage from injured seedlings might have resulted from damage to the plasma membrane and possibly also to the tonoplast, which affects membrane structure, physical integrity, and composition (Levitt, 1980). Lyons and Raison (1970) suggested that the transition of a membrane from a flexible liquid crystalline structure to a solid-gel was the primary effect of low temperature. Recently, Kodama et al. (1994) produced transgenic tobacco containing an ω-3 fatty acid desaturase gene (fad7) from *Arabidopsis* that increased resistance to chilling stress. When 5-day-old green mungbean seedlings were exposed to 4°C, they suffered chilling injury. These results agree with those from previous studies (Ma et al., 1990; Chen et al., 1991). Prolonged exposure to low temperatures increased the leakage of solutes, such as soluble sugars, free amino acids and cations, from the leaves (Figures 2, 3 and 4).

There was a positive relationship between the conductivity and the amount of cations (K^{+}, Mg^{++} and Ca^{++}) in

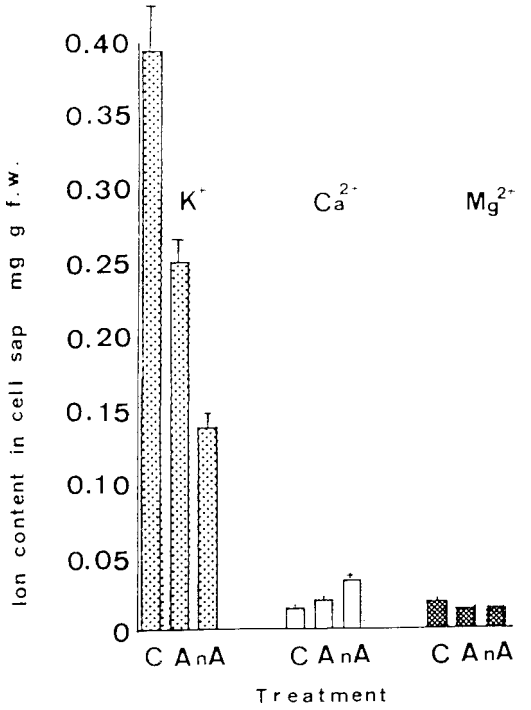

Figure 6. Concentrations of K^{+}, Mg^{++} and Ca^{++} in the cell sap of leaf discs from mung bean seedlings subjected to different temperature treatments. The experimental procedures are the same as in Figure 4. Concentrations of K^{+}, Mg^{++} and Ca^{++} in the sap were measured. Vertical lines represent standard errors (n=3). C: Control (28°C, 7d); A: Acclimated (28°C, 5d → 10°C, 2d → 4°C, 2d); nA: Unacclimated (28°C, 5d → 4°C, 2d).

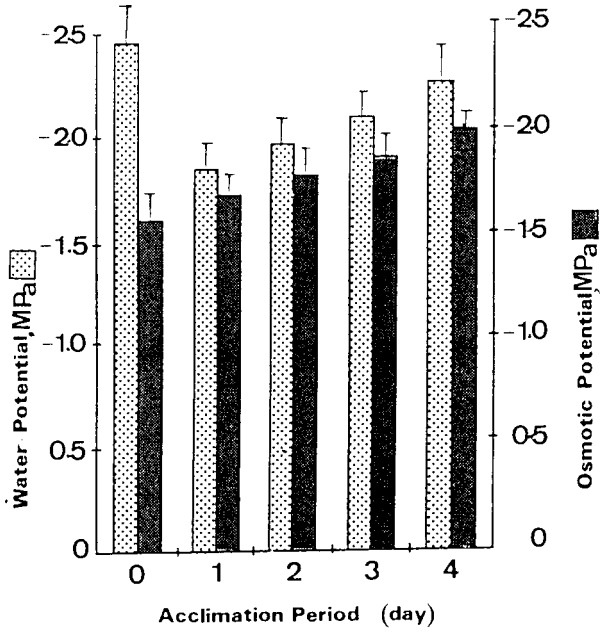

Figure 7. Water potential and solute potential of the leaves of mungbean seedlings given the 4°C chilling or 10°C cold acclimation treatment. Five day-old mungbean seedlings were treated at 10°C for various lengths of time. The water potential and osmotic potential in the leaf discs were measured. The water potential and osmotic potential in the leaf discs from the 28°C control were -1.427 MPa and -1.206 MPa, respectively. Vertical lines represent standard error (n=3).

Table 2. Water potential, osmotic potential, and conductivity of the cell leakage from leaf discs taken from mungbean seedlings subjected to different root/shoot temperature treatments. Five day-old mungbean seedlings were subjected to water culture and then subjected to different root/shoot temperature treatments for 2 days. Water potential, osmotic potential in the cell sap and conductivity of the cell leakage from leaf discs were measured. Data are expressed as means ± SE (n=3).

Treatment (°C)		Water potential	Osmotic potential	Conductivity
Root temp.	Leaf temp.	(Mpa)	(Mpa)	(us)
10	10	-1.667±0.052	-1.468±0.037	10.2±0.1
28	10	-1.562±0.050	-1.356±0.035	8.7±0.1
28	28	-1.458±0.045	-1.208±0.003	8.6±0.1
28	4	-1.823±0.045	-1.450±0.035	186.8±6.6
4	4	-2.292±0.074	-2.007±0.005	761.0±22.0

Table 3. Concentration of free amino acids, soluble sugars, and cations in the cell sap of mungbean seedling leaves after 48 h of different root/shoot temperature treatments. The experiment procedures are the same as in Table 2. Data are expressed as means ± SE (n=3).

Treatment (°C)		Free amino acids	Soluble sugars	Cation content (mg/g fresh weight)		
Root temp.	Leaf temp.	(mg/g fresh weight)		K^+	Ca^{2+}	Mg^{2+}
10	10	1.43±0.03	22.53±0.75	0.293±0.008	0.015±0.004	0.013±0.002
28	10	2.77±0.06	20.70±0.47	0.334±0.014	0.011±0.001	0.015±0.002
28	28	2.93±0.10	16.55±0.50	0.369±0.008	0.010±0.002	0.015±0.003
28	4	2.06±0.04	32.31±1.31	0.194±0.011	0.017±0.002	0.014±0.002
4	4	1.22±0.04	38.44±1.38	0.083±0.005	0.020±0.002	0.011±0.001

leakage. The large amount of K^+ in leakage may be due to the high concentration of K^+ in leaf tissue for stomatal movement and /or oxidative phosphorylation dysfunction (Lardy, 1952) after chilling injury. Pretreatment of seedlings at l0°C for 3 days reduced the severity of chilling injury at 4°C as evidenced by the low cation concentrations in leakage compared with those from unacclimated leaf tissues.

Calcium ions are a primary physiological transducer of chilling injury (Minorsky, 1985). Chilling caused an increase in Ca^{++} concentration in the cytoplasm (Figure 6). These increases could cause several physiological changes, such as 1) inhibition of ethylene biosynthesis (Lieberman and Wang, 1982), 2) inhibition of photosynthesis and 3) activation or repression of phosphatase activity leading to a change in the phosphorylation stature of another protein. These processes provide further signal transduction pathways for chilling responses (Hughes and Dunn, 1996). In addition, Mg^{++} and Ca^{++} are essential for the structural integrity and normal differential permeability of membranes (Christiansen et al., 1970; Epstein, 1972). After chilling stress, the amounts of Ca^{++} or Mg^{++} in the leakage from unacclimated leaves were higher than in the leakage from acclimated leaves.

Cold acclimation proteins may play a physiological role similar to that of heat shock proteins (HSPs) (Key et al., 1981) in protecting organisms from injury at low temperatures (Chen et al., 1991). HSPs in cells are vital for increasing thermotolerance (Lin et al., 1984; Lin et al., 1985; Chou et al., 1988; Jinn et al., 1989; Jinn et al., 1995; Yeh et al., 1997). Heat shock proteins are associated with plasmalemma (Lin et al., 1984) and are thought to be physiologically important in reducing cellular leakage of solutes in soybean seedlings (Lin et al., 1984). Like HSPs, cold acclimation proteins are associated with nuclei, mitochondria, and ribosomes (Chen, unpublished data), which may explain why lower amounts of amino acids, soluble sugars, and ions were found in the leakage of chilled, cold acclimated seedlings than in that of chilled, unacclimated seedlings.

Exposure for 24 h, of 7-day-old, green mungbean seedlings to light at 4°C, caused wilting of the leaves, probably from solute leakage. A high concentration of solutes in the cell sap should lower the osmotic potential so that more water can be absorbed, via the roots, into the seedlings (Levitt, 1939; Levitt, 1957).

At temperatures of 0°C to 10°C, hydrophobic bonding in proteins decreases, causing conformation changes in protein structure (Adva and Waisel, 1975) and irreversible injury to plants. The 10 amino acids that increased in the tissues of acclimated seedlings were all polar (Table 1) and may be involved in the maintenance of water content and increases in enzymatic activity and tissue metabolism.

Root temperature has important affects on the growth and absorption of water and mineral nutrients. Low root temperature may result in decreased absorption of water and mineral nutrients by the roots. Because the viscosity of cytoplasm and the density of water at 4°C are higher than at 28°C, growth and physiological activities, such as cellular metabolism and biosynthesis of plant hormones,

in the roots should be reduced at low temperatures. Therefore, when seedlings (root and stem) were grown at 4°C, they suffered severe chilling injury, including leaf wilting. On the other hand, when the root temperature was kept at 28°C and the shoot was exposed to 4°C, chilling injury was reduced to a minimum, indicating the importance of root temperature. During cold acclimation, some physiological and biochemical processes change, including solute concentrations in the cell sap and gene expression (Hughes and Dunn, 1996). Research is under way to understand the molecular and genetic basis of cold tolerance and the functions of proteins induced during cold hardening.

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冷馴化與根溫對綠豆幼苗低溫傷害的影響

張孟瑤　陳淑玲　李中芬　陳益明

國立台灣大學植物系

綠豆幼苗在 4℃ 溫度下經二天即產生不可逆的寒害而枯死。分析經 4℃ 低溫處理過的組織滲透物中之陽離子，發現 K^+ 離子濃度比 Ca^{++} 及 Mg^{++} 離子高出 7-10 倍。若先於 10°C 下作低溫馴化可減少在 4℃ 低溫下所造成的傷害。在 10℃ 溫度下經 2 至 3 天的先前處理，其滲透物中的導電度、可溶性糖的濃度、游離胺基酸及陽離子（K^+, Mg^{++} 及 Ca^{++}）都顯著減少。將幼苗種於根溫 28°C 及地上部 4°C 下與全株都在 28°C 生長的對照組相比較，由結果得知上述處理組的幼苗並無明顯的傷害。在 28°C 根部／4°C 地上部生長的處理組幼苗，其溶質勢（solute potential）與水勢（water potential）都與 28°C 對照組植物相似，且細胞液（cell sap）內的胺基酸、陽離子（K^+, Mg^{++} 及 Ca^{++}）濃度亦與 28°C 對照組相同。

關鍵詞：綠豆；寒害；低溫馴化；根溫；細胞液；滲漏物；導電度。